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Original Research

Different Glyphosate Phytotoxicity of Seeds and Seedlings of Selected Plant Species

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Abstract

The aim of this study was to compare the physiological responses of six plant species (popular crops or plants recommended as indicators of soil pollution) to a wide range of glyphosate concentrations (0, 1, 3, 7, 10, 40, 80, 120, 180, 240, 400, 750, 1,000, 1,500, 1,700 and 2,000 μM). Percent germination, root length, seedling dry mass and myo-inositol content, as well as seedling leachate electroconductivity were determined in *Lepidium sativum, Sinapis alba, Sorghum saccharatum, Brassica napus, Lupinus luteus* and *Avena sativa*.

Percent seed germination, seedling dry mass and electroconductivity of seedling leachates were not clearly affected by the herbicide and could not be used as indicators of its phytotoxicity. An metabolite induced by abiotic stresses in many plants, *myo*-Inositol, was very strongly stimulated by glyphosate at doses above 10 or 40 μM, depending on plant species.

The sensitivity of analyzed plants to glyphosate, as manifested by root length, differed clearly. In *Avena sativa* the relationship between root length and glyphosate concentration was fairly linear over a wide range of herbicide doses (up to 240-400 μM). The most distinct drop in root growth at low herbicide doses was visible in *Sorghum saccharatum*.

The results show that a mild stress affecting root length may not clearly modify seedling *myo*-inositol levels, that respond distinctly to stronger stresses. Not all indicator plants are equally suitable for analysis of biological activity of glyphosate residues. *Sorghum saccharatum* seems particularly sensitive.

Keywords: glyphosate, electroconductivity, *myo*-inositol, root growth test, seed germination

Introduction

Glyphosate (N-(phosphonomethyl)-glycine) is a nonselective, systemic herbicide that controls most annual and perennial weeds [1]. It is used in agriculture, fruit farming, vegetable production, and forestry but also in landscape management for removal of undesirable vegetation from aquatic and urban ecosystems. Recently it has been increasingly used in production of genetically modified organisms (e.g. soybean, cotton and maize) [2]. Consequently, glyphosate application has increased in recent years [3-4].

The U.S. Environmental Protection Agency [5] classifies glyphosate as category IV – the least toxic herbicide. It is also considered not carcinogenic and not mutagenic for humans and other mammals [6-8, 9]. However, long-term exposure to glyphosate can cause toxicity and ecotoxicity. Some authors caution against the use of this herbicide near water reservoirs since it has a toxic effect on tadpoles, adult frogs and fish [10-11].

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Glyphosate applied to crops in recommended or smaller doses as preharvest treatment can lead to seed deformation and yield decrease [12-14], that might be significant in some seed production systems. Used as a desiccant during the plant maturation phase, glyphosate can affect levels of indole-3-acetic acid (IAA), the main endogenous auxin, and thus inhibit germination and seedling growth [15]. The half-life of glyphosate in soil is only 10 to 100 days (47 days on average), according to Hornsby et al. [16] and according to Monsanto [1] an average half-life is 32 days. Keeping in mind the above considerations, determination of glyphosate residues in soil may be important from both agricultural and ecotoxicological viewpoints.

Biotests, including those based on seedlings of higher plants, are considered inexpensive and simple methods for assessment of herbicide residues in soil. The aim of the present study was to compare the utility of some plant species as components of such phytotests and some physiological parameters as criteria of analyses.

Material and Methods

Seed Germination and Root Growth Test

Seeds of oilseed rape (*Brassica napus*), white mustard (*Sinapis alba*), yellow lupin (*Lupinus luteus*), cress (*Lepidium sativum*), oats (*Avena sativa*) and sorghum (*Sorghum saccharatum*) were germinated for three and six days using PHYTOTOXKIT™ (MicroBio Test Inc., Belgium). Germination was carried out in controlled climatic conditions with temperature set at 25ºC and 90% RH humidity, in darkness. Germination was scored after 3 and 6-d imbibition, when the radicle had emerged from the testa. Ninety ml of reference soil (sand, vermiculite, peat 1:0.3:1, v/v/v, a standard mixture provided by the PHYTOTOXKIT™ manufacturer) were placed in plastic microbiotest plates. The soil was covered with Whatman No. 1 filter-paper and watered. The amount of water (27 ml) needed to hydrate soil sample in each biotest plate was calculated according to recommendation of the PHYTOTOXKIT™ manufacturer. The soil samples in glyphosate treatment plates were hydrated with 27 ml aqueous solutions of glyphosate (Roundup Ultra 360 SL containing 360 g/L active principle) at final concentrations: 1, 3, 7, 10, 40, 80, 120, 180, 240, 400, 750, 1000, 1500, 1700 or 2000 µM. The control plants were watered with pure distilled water. The root length was estimated using Image Tool for Windows. The effective concentration causing a 50% response (EC_{50}) was calculated for inhibition of root growth. Dry and fresh mass and electroconductivity were determined according to ISTA [17].

myo-Inositol Content

myo-Inositol content in the roots was analyzed by GC chromatography according to Piotrowicz-Cieślak [18]. Tissues (30-60 mg fresh mass) were homogenized in ethanol:water, 1:1 (v/v) containing 300 µg phenyl-α-D-glucose as internal standard. The homogenate was transferred to a 1.5 ml microfuge tube, heated at 75ºC for 30 min to inactivate endogenous enzymes and centrifuged at 15,000 g for 20 min. The supernatant was passed through a 10,000 MW cut-off filter (Lida and Kenosha WI USA). Aliquots of 0.3 ml filtrate were transferred to silylation vials and evaporated to dryness under a stream of nitrogen. Dry residues were derived with 300 µl of silylation mixture (trimethylsilylimidazole:pyridine, 1:1, v/v) in silylation vials (Supelco) at 70ºC for 30 min and then cooled at room temperature. One µl carbohydrate extract was injected into a split-mode injector of a Shimadzu GC-14A gas chromatograph equipped with flame ionization detector and Shimadzu C-R6A integrator. myo-Inositol was analyzed on a DB-1 capillary column (15 m length, 0.25 mm ID, 0.25 µm film thickness, J&W Scientific) and identified with internal standard. The metabolite concentrations were calculated from the ratios of sample peak area to the internal standard peak area. Quantities of *myo*-inositol were expressed as means ± SD for 3-5 replications of each treatment.

Statistical Analysis

The experiment was carried out in nine replicates. The results were statistically evaluated using analysis of variance (F test) for two factor experiments (split-plot). The mean values of the plots were compared using q SNK test (Student-Newman-Keuls).

Results

After six days 97% of seeds of all tested plants germinated in control soil and 85% in the soil with maximum glyphosate concentration (Fig. 1). As much as 93% of the seeds germinated in soil with 7 µM glyphosate.

In all species except *Sinapis alba* and *Lepidium sativum* the root length measured in control plants after three days was nearly twice shorter than after six days. A similar pattern was obtained for treatments with low concentrations of glyphosate (1 to 10 µM in *B. napus*, *L. luteus* and *Sorghum saccharatum*; 1 to 80 µM in *A. sativa*). In *S. alba* and *L. sativum* root growth between three and six days of analysis increased by no more than 20%. In both these plants, however, similarly to the above species, glyphosate doses above 10 µM prevented root length increase between three and six days of analysis (Fig. 1).

Glyphosate at concentrations 240 µM-2,000 µM caused a similar degree of root growth inhibition measured against control seedlings (on average across species, 81% or 88%, respectively). At these herbicide concentrations seedling root length did not show any increase between three and six days of germination. Noteworthy, in *Lepidium sativum* seedlings glyphosate concentrations within the range 1-7 µM caused a slight stimulation of root growth.

Glyphosate concentration effectively inhibiting root growth in 50% plants (EC_{50}) for *Brassica napus, Lupinus*

luteus, Lepidium sativum, Avena sativa and *Sorghum saccharatum* was 35, 25, 30, 30, 110 and 22 μM, respectively. Although the EC_{50} values were similar for all plants except *A. sativa, S. saccharatum* and *L. luteus* were found to be the least sensitive among analyzed plants.

Root dry mass measured six days after glyphosate application increased slightly but consistently with the increase of glyphosate concentration, so that in all species, except *Lepidium sativum*, it exceeded the value recorded for control seedlings up to 1.4 times and at the highest herbicide concentration it reached 20% (Fig. 2). *Lepidium sativum* was characterised by low dry mass of roots in control seedlings (7.5%) and high stimulation of dry mass by glyphosate (1.9 times at 2,000 μM concentration). Similarly to dry mass, seedling leachate electroconductivity increased steadily, although at a lower rate (Fig. 2).

Six days after glyphosate application the *myo*-inositol content in roots of control seedlings was highest in *Brassica napus* (0.26 mg/g dry mass (d.m.) and lowest in *Avena sati-*

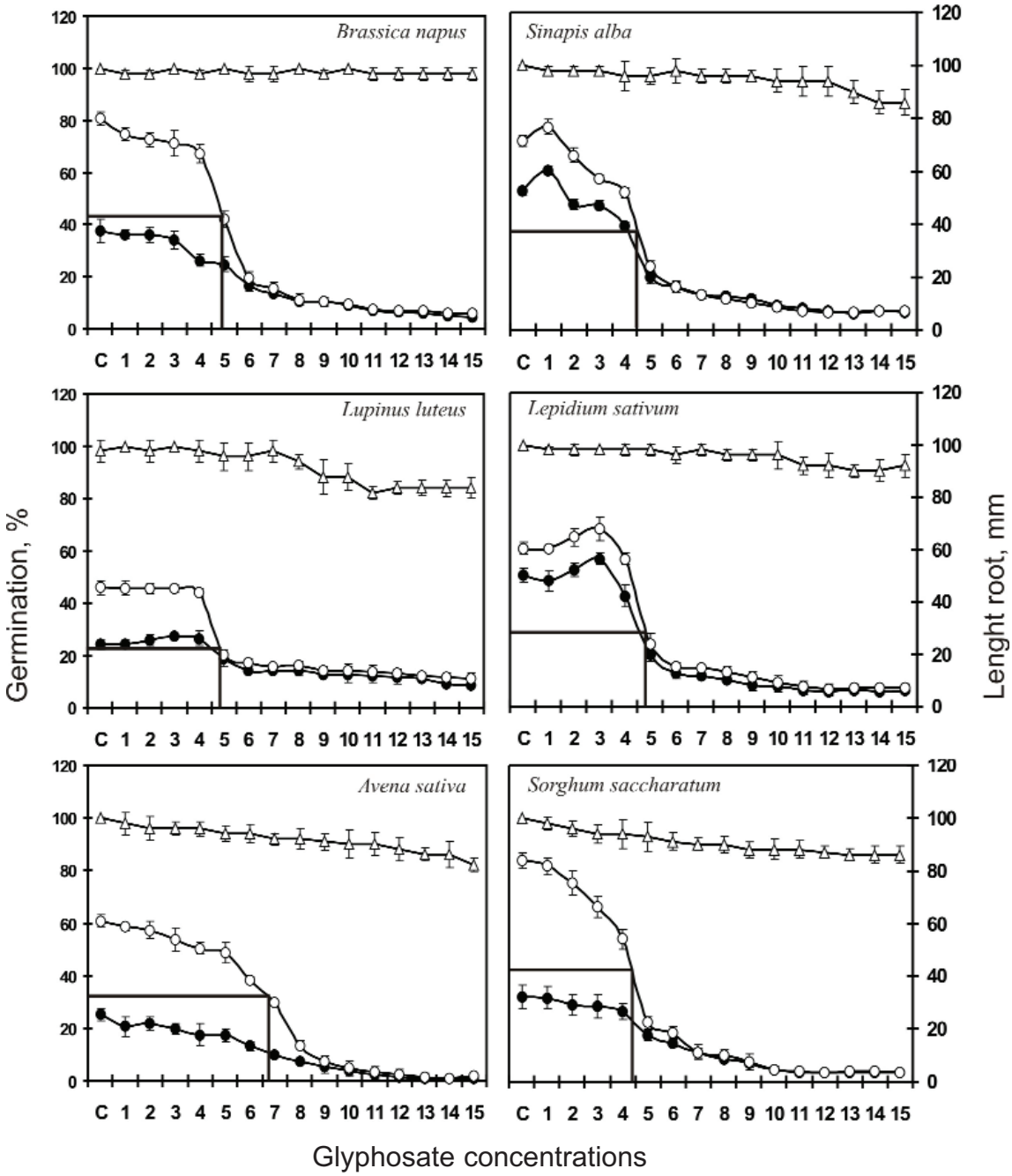

Fig. 1. Seed (Δ) germination [%] and root length [mm] of *Brassica napus, Sinapis alba, Lupinus luteus, Lepidium sativum, Avena sativa* and *Sorghum saccharatum* growing for three (●) and six (○) days in soil supplemented with different glyphosate concentrations (c – control, 1-1 μM, 2-3 μM, 3-7 μM, 4-10 μM, 5-40 μM, 6-80 μM, 7-120 μM, 8-180 μM, 9-240 μM, 10-400 μM, 11-750 μM, 12-1,000 μM, 13-1,500 μM, 14-1,700 μM and 15-2,000 μM). Bars illustrate EC_{50}. Data points represent the means ± SD for nine replicate samples.

va (0.036 mg/g d.m.). With rising amounts of glyphosate, increased myo-inositol content was recorded. In *Lepidium sativum* this stimulation started at the lowest glyphosate dose (above 7 μM). Above 40 μM glyphosate was needed to obtain *myo*-inositol rise in *Lupinus luteus, Brassica napus* and *Synapis alba*.

Discussion

Seed germination and seedling growth are increasingly used as the basis of biotests that allow simple and inexpensive analysis of biological activity of environmental pollutants, including herbicides, cyanobacterial metabolites,

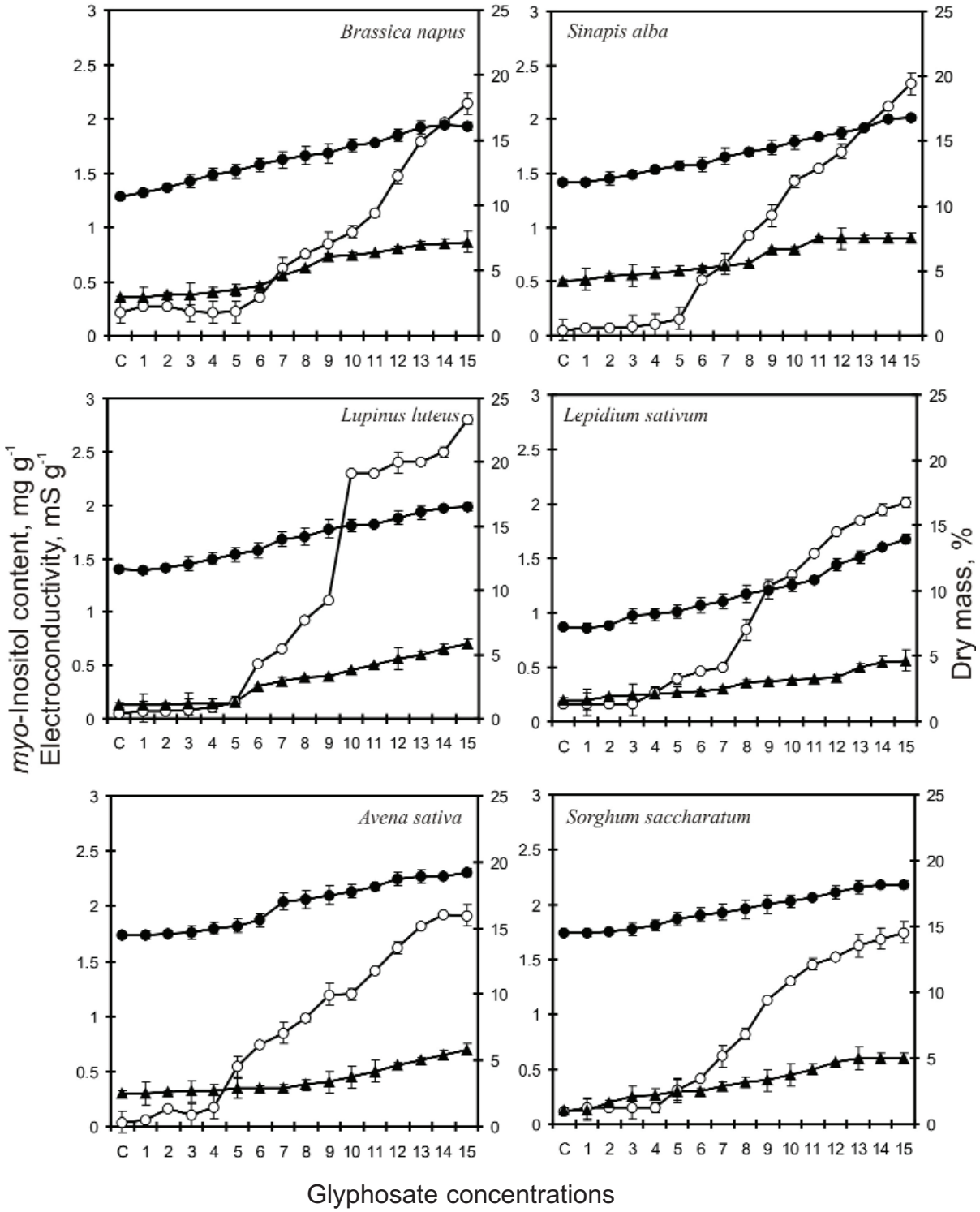

Fig. 2. Content of *myo*-inostol (○, [mg g^{-1} dry mass]) in roots, electroconductivity (▲, [mS fresh $mass^{-1}$]) and dry mass (●, [%]) of *Brassica napus, Sinapis alba, Lupinus luteus, Lepidium sativum, Avena sativa* and *Sorghum saccharatum* seedlings growing for six days on soil supplemented with different glyphosate concentrations (c – control, 1-1 μM, 2-3 μM, 3-7 μM, 4-10 μM, 5-40 μM, 6-80 μM, 7-120 μM, 8-180 μM, 9-240 μM, 10-400 μM, 11-750 μM, 12-1,000 μM, 13-1,500 μM, 14-1,700 μM and 15-2,000 μM). Data points represent the means ± SD for nine replicate samples.

antitumor medicines and even radioactive isotopes [19-20]. In many cases these biotests offer lower sensitivity than tests based on small animals (e.g. *Drosophila melanogaster, Daphnia pulex, Tubifex tubifex*). Nevertheless, the use of plant models seems quite reasonable in studies of compounds known for high phytotoxicity [21]. Usually *Sinapis alba, Lepidium sativum* or *Sorghum saccharatum* are chosen for such tests. In this study, it was shown that seedlings of some crop species – *Brassica napus* and *Avena sativa* - are affected by glyphosate herbicide at lower doses than those inhibitory for the above-mentioned biotest plants. Even the dose 7-fold lower than that recommended in agronomical practice (7 μM, i.e. 3.0 L /ha Roundup Ultra 360 SL [22]) clearly inhibited root growth in *B. napus* and *A. sativa*, while it did not suppress root elongation in *Lepidium sativum* and it even increased root length in *Sinapis alba*. For glyphosate concentrations within the range 1-40 μM the sharpest drop in root length occurred in *Sorghum saccharatum,* which confirms the value of this plant as a herbicide sensor plant in biotests. However, even the highest glyphosate concentration did not cause visible symptoms of seedling damage (other than growth inihibition) in any analyzed species (Fig. 3).

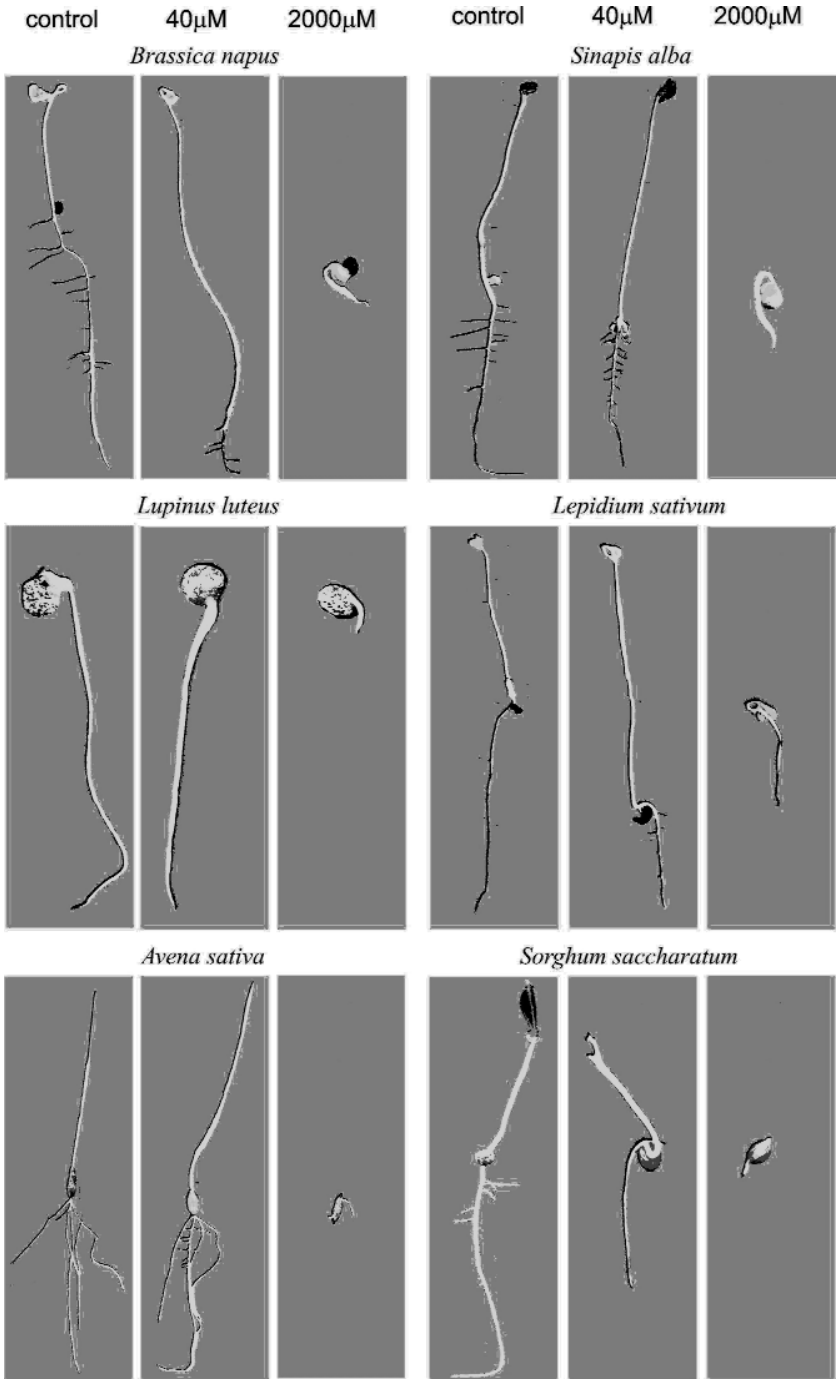

Fig. 3. *Brassica napus, Sinapis alba, Lupinus luteus, Lepidium sativum, Avena sativa* and *Sorghum saccharatum* seedlings growing for six days in soil supplemented with 0 μM, 40 μM or 2,000 μM glyphosate.

Effective concentration causing a 50% response (EC_{50}) of root growth after six days for *Sinapis alba, Sorghum saccharatum, Brassica napus* and *Avena sativa* was 25, 22, 35 and 110 μM, respectively. Comparing these results to published data, it should be noted that the values obtained in this study (for all species except *Avena sativa*) were rather low – reported glyphosate EC_{50} for *Lemna gibba* was 73.3 μM and for phytoplankton (green alga) 60.3 μM [23].

Physiological parameters other than root length (measured after six days of germination) were much weaker indices of herbicide activity in all tested species. Similar results were obtained by Torres et al. [24] and Kohata et al. [25], who found that glyphosate inhibits shoot growth but not seed germination in watergrass, transgenic and non-transgenic soybean. Roots and seedlings are more sensitive to glyphosate activity than fully developed plants [26].

The electrical conductivity method was successfully used to ascertain the degree of freezing injury [27]. In this study suitability of this test was analyzed for evaluation of seedling damage after glyphosate application. Electroconductivity of seedlings growing under control conditions amounted to 0.5 mS/g f.m. and increased to 3 mS/g f.m. in seedlings growing at 2,000 μM glyphosate.

The *myo*-inositol is commonly considered a stress metabolite in plants, stimulated by drought and salinity [28, 29]. Although there are no published data on herbicides effect on *myo*-inositol levels in plants, a desiccative action of herbicides is well established [30]. It was assumed, then, that plant responses to herbicides' might involve mechanisms active under drought stress. Interestingly, the experimental data did not fully corroborate this hypothesis. The level of *myo*-inositol in plants treated with glyphosate increased, but only when the herbicide had been used at very high concentrations (above 10-40 μM). The content of free *myo*-inositol in plant roots is generally relatively low. In roots of Japanese persimmon (*Diospyros kaki*) *myo*-inositol could be found in amounts ranging from 0.9 to 1.2 mg/g d.m. (compared to 50 m/g d.m. in *Eucalyptus* seedlings growing in xeric ecosystems) [31, 28]. Furthermore, plant roots contain a transport system protecting them from high *myo*-inositol content by exuding it to the soil [32].

Conclusions

1. Among analysed plants *Sorghum saccharatum* showed very marked responses to glyphosate at concentrations 1-120 μM and could be used as bioindicator of soil residues of this herbicide.
2. The root length measured after six days of germination was the best index of herbicide stress.
3. *myo*-Inositol increase in roots of studied plants occurred only at very high glyphosate concentrations (above 10-40 μM).

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